Journal of Neurochemistry
Lippincott–Raven Publishers, Philadelphia

Rapid Communication

A Hydrophilic Peptide Comprising 18 Amino Acid Residues of the Prosaposin Sequence Has Neurotrophic Activity In Vitro and In Vivo

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Abstract: Prosaposin, a 517-amino-acid glycoprotein, not only acts as the precursor of saposin A, B, C, and D but also possesses neurotrophic activity to rescue hippocampal CA1 neurons from ischemic damage in vivo and to promote neurite extension of neuroblastoma cells in vitro. Recently, the trophic activity of prosaposin on human neuroblastoma cells has been shown to reside in the NH_2-terminal hydrophilic sequence (LIDNNRTEEILY) of the human saposin C. Here we show that prosaposin, saposin C, and a peptide comprising the 18-amino-acid sequence (18-mer peptide; LSELIINNATEELLIKGL) located in the NH_2-terminal hydrophilic sequence of the rat saposin C-domain promoted survival and neurite outgrowth of cultured rat hippocampal neurons in a dose-dependent manner. Moreover, infusion for 7 days of the 18-mer peptide into the lateral ventricle of gerbils, starting either 2 h before or immediately after 3 min of forebrain ischemia, protected ischemia-induced learning disability and hippocampal CA1 neuronal loss. Thus, we ascribe the in vitro and in vivo trophic actions of prosaposin on hippocampal neurons to the linear 18-mer sequence and raise the possibility that this peptide can be used as an agent for the treatment of forebrain ischemic damage. **Key Words:** Prosaposin—Neurotrophic activity—Active sequence—Hippocampal neurons—Ischemia.
J. Neurochem. **66,** 2197–2200 (1996).

Prosaposin, the precursor of saposins A, B, C, and D, which activate sphingolipid hydrolases in lysosome, is a 517-amino-acid glycoprotein (O'Brien and Kishimoto, 1991; Kishimoto et al., 1992). Besides its role as the precursor of saposins in lysosome, prosaposin itself exists as a secretory protein in human milk, CSF, and seminal plasma (Hineno et al., 1991; Kondoh et al., 1991; Hiraiwa et al., 1993). Prosaposin is abundant in the brain and muscle, whereas processed saposins are predominantly found in the spleen, liver, and kidney (O'Brien et al., 1988; Sano et al., 1989). In the brain, prosaposin is localized exclusively to certain neurons and nerve fibers. Furthermore, prosaposin-containing neural elements are abundant in the CA1 field of the hippocampus (Kondoh et al., 1993), which is known to be vulnerable to ischemic insult. Recently we demonstrated that prosaposin has a potent ability to rescue hippocampal neurons from lethal ischemic damage in vivo (Sano et al., 1994), raising the possibility of prosaposin as a neurotrophic factor candidate. The pivotal role of prosaposin in the brain has also been indicated by Harzer et al. (1989) and Schnabel et al. (1992), who reported that prosaposin deficiency causes fetal neurological deficits. O'Brien et al. (1994, 1995) demonstrated that prosaposin stimulates neuritogenesis and increases choline acetyltransferase activity in neuroblastoma cells and that the neurotrophic activity resides, at least in part, in an amino-terminal 12-residue peptide encompassing the hydrophilic region of the human saposin C domain. These findings encouraged us to investigate the in vitro and in vivo neurotrophic activities of another peptide comprising the hydrophilic region of the rat saposin C domain.

In the present study, we examined the effects of prosaposin, saposins A, B, C, and D, and a new synthetic peptide with 18-amino-acid residues that contain the hydrophilic sequence of the rat saposin C domain on cultured hippocampal neurons. Furthermore, to investigate the activity of the peptide in vivo, we studied whether or not the peptide prevented ischemia-induced learning disability and hippocampal CA1 neuronal loss in gerbils.

MATERIALS AND METHODS

Materials

Saposins A, B, C, and D were isolated from bovine spleen, and prosaposin was purified from human milk as previously described (Sano et al., 1988; Kondoh et al., 1991). An 18-mer peptide (LSELIINNATEELLIKGL) was chemically synthesized in response to our purchase order (Sawady Technology, Tokyo, Japan).

Culture study of embryonic day 17 rat hippocampal neurons

Neurons were prepared separately from the rat hippocampi of 17-day embryos (Hatanaka et al., 1988) and cultured on plastic coverslips according to the procedures of Akaike et al. (1991) with slight modifications. In brief, the hippocampi were treated with trypsin and digested with DNase I (Sigma).

Resubmitted manuscript received January 15, 1996; accepted January 19, 1996.

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Abbreviations used: BSA, bovine serum albumin; MAP 2, microtubule-associated protein 2; PBS, phosphate-buffered saline.

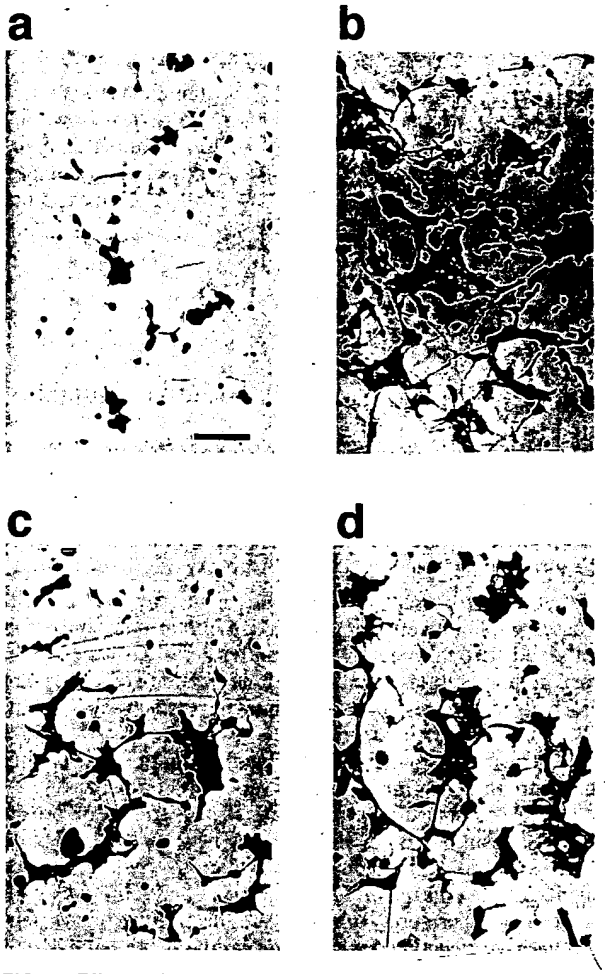

FIG. 1. Effects of prosaposin and its derivatives on hippocampal MAP 2-positive neurons in culture: (**a**) vehicle, (**b**) prosaposin (200 ng/ml), (**c**) saposin C (400 ng/ml), and (**d**) 18-mer peptide (160 ng/ml). Bar = 100 μm.

Hippocampal neurons were sedimented after addition of Dulbecco's modified Eagle's medium (Gibco) containing 10% fetal calf serum. The hippocampal neurons (1×10^5) were cultured on a poly-L-lysine-coated plastic coverslip (11 × 11 mm) in Dulbecco's modified Eagle's medium with fetal calf serum for the initial 24 h. Thereafter the medium was replaced with a serum-free medium (Hatanaka et al., 1988) containing various concentrations of prosaposin, saposins A, B, C, and D, or the 18-mer peptide, and then the neurons were further cultivated for 48 h; the hippocampal neurons incubated with the serum-free medium alone were used as control. Microtubule-associated protein 2 (MAP 2)-positive cells were counted in triplicate from different portions of each well, and the ratio of the number of MAP 2-positive cells bearing neurites three times longer than the cell diameter to the number of all MAP 2-positive cells was calculated.

In vivo ischemia study

The in vivo study of ischemia was performed as previously described (Sano et al., 1994; Wen et al., 1995*a,b*). Male Mongolian gerbils weighing 70–80 g were anesthetized with halothane and nitrous oxide. An osmotic minipump (Alza Corp., Palo Alto, CA, U.S.A.) was implanted subcutaneously into the back of each animal, and a needle from the minipump was placed in the left lateral ventricle. The 18-mer peptide dissolved in phosphate-buffered saline (PBS) at a dose of 7 or 20 ng/day was continuously infused for 7 days into the lateral ventricles of gerbils in which 3-min forebrain ischemia had been induced. Control animals received saline infusion or infusion of PBS with 0.5% bovine serum albumin (BSA). The infusion was started 2 h before or immediately after the ischemic insult. In the latter cases, a high dose of the synthetic peptide (0.8 or 8.0 ng/μl dissolved in 5 μl of PBS) was slowly infused with a syringe, and then 20 ng/day of the peptide was continuously infused by an osmotic minipump.

Seven days later, the gerbils were trained in a conventional step-down passive avoidance apparatus. Each animal was initially placed on the safe platform on a training day. When the gerbil stepped down onto the grid floor, it received a foot shock. Although the gerbil went repeatedly up and down between the platform and the grid, it eventually remained on the platform. This training session lasted for 300 s. Twenty-four hours later, the gerbil was again placed on the same platform while the shock generator was turned off, and the response latency, i.e., the time until it stepped down to the grid floor, was measured. This test session also lasted for 300 s (Sano et al., 1994; Wen et al., 1995*a,b*). After passive avoidance experiments, the animals were injected with bromophenol blue through the needles of the minipumps to show dye diffusion into the cerebral ventricles.

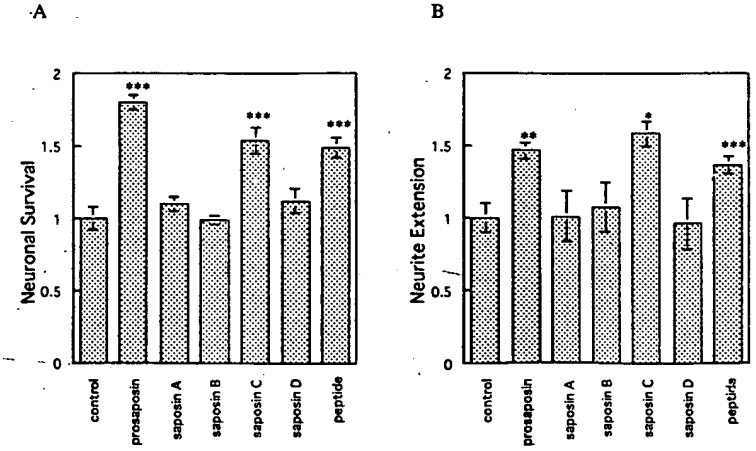

FIG. 2. Neurotrophic effects of prosaposin, saposins, and an 18-mer peptide on embryonic day 17 rat hippocampal neurons: (**A**) neuronal survival and (**B**) neurite outgrowth. MAP 2-positive cells were counted in triplicate from different portions of each well, and the ratio of the number of MAP 2-positive cells bearing neurites three times longer than the cell diameter to the number of all MAP 2-positive cells were calculated. Doses were as follows: prosaposin, 200 ng/ml; saposins A–D, 400 ng/ml; and the 18-mer peptide, 160 ng/ml. Data are mean ± SE (bars) values (n = 5–10). $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

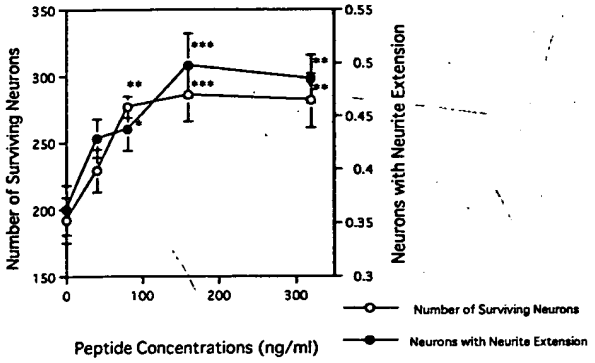

FIG. 3. Dose-dependent effects of the 18-mer peptide on rat hippocampal neurons. Neuronal survival and neurite extension were analyzed as described in Fig. 1. Data are mean ± SE (bars) values (n = 4–5). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

Then they were anesthetized with chloral hydrate and perfused transcardially with 4% paraformaldehyde/1% glutaraldehyde in 0.1 *M* phosphate buffer (pH 7.4). After perfusion the dorsal hippocampus was removed, three sections 100 μm thick transverse to the axis of the dorsal hippocampus were cut for electron microscopy, and the remaining hippocampus was embedded in paraffin and cut into 5-μm serial sections that were stained with 0.1% cresyl violet. Viable neurons in the hippocampal CA1 field were counted and recorded as neuronal density per 1 mm of linear length.

Statistics

The effects of prosaposin, saposins, and the 18-mer peptide on cultured neurons were evaluated by *F* test and Student's *t* test, and data from the in vivo experiments were evaluated by *F* test and Student's *t* test or Mann–Whitney U-test, which compared the peptide-treated groups with the vehicle or PBS/BSA-treated control groups.

RESULTS AND DISCUSSION

In the present study, we examined the neurotrophic activities of prosaposin, saposins, and a new 18-mer peptide containing the hydrophilic sequence of the rat saposin C domain on embryonic hippocampal neurons. As shown in Fig. 1, the number and neurite extension of MAP 2-positive neurons were promoted in prosaposin-, saposin C-, or the 18-mer peptide-treated culture. Prosaposin, saposin C, or the 18-mer peptide stimulated significantly neuronal survival and neurite outgrowth, whereas saposins A, B, and D were ineffective (Fig. 2). The concentrations of prosaposin, saposin C, and the peptide required for supporting neuronal survival were almost the same as those for stimulating neurite outgrowth, and they acted in a dose-dependent manner. Data for the 18-mer peptide are shown in Fig. 3 (prosaposin and saposin C, data not shown). Half of the peak effective dose of prosaposin was 1.4 n*M* (100 ng/ml); a five times higher molar concentration of saposin C or 20 times higher concentration of the 18-mer peptide was needed to yield the same action as that of 1.4 n*M* prosaposin. Similar neurotrophic effects of prosaposin and its partial peptide (LIDNNRTEEILY) on human neuroblastoma cells were observed at concentrations of the same order of magnitude (O'Brien et al., 1994).

It is not always possible to compare the neurotrophic activity of prosaposin or the 18-mer peptide with that of other neurotrophic factors so far examined. However, the optimal concentrations of prosaposin, saposin C, and the 18-mer peptide for supporting cultured hippocampal neurons were similar to or lower than the concentration of basic fibroblast growth factor (Ohsawa et al., 1993; Nakata et al., 1993). Moreover, the neurotrophic sequence in the saposin C domain is different from that of the major glucocerebrosidase-stimulating region (Sano et al., 1992; Weiler et al., 1995), reinforcing the notion that prosaposin not only acts as the precursor of saposins A, B, C, and D to activate sphingolipid hydrolases but also subserves neurotrophic function.

To see the neurotrophic activity of the 18-mer peptide in vivo, we used ischemic gerbils, which showed an apparent decline in response latency in the passive avoidance test and a loss of hippocampal CA1 neurons, in comparison with sham-operated animals (Sano et al., 1994; Wen et al., 1995*a,b*). Continuous peptide infusion into the lateral ventricle, which started 2 h before forebrain ischemia and lasted for 7 days, caused a dose-dependent prolongation in response latency in the step-down passive avoidance task, when compared with the vehicle- and PBS/BSA-infusion group: 20 ng/day of peptide treatment, 203 ± 20 s; vehicle infusion,

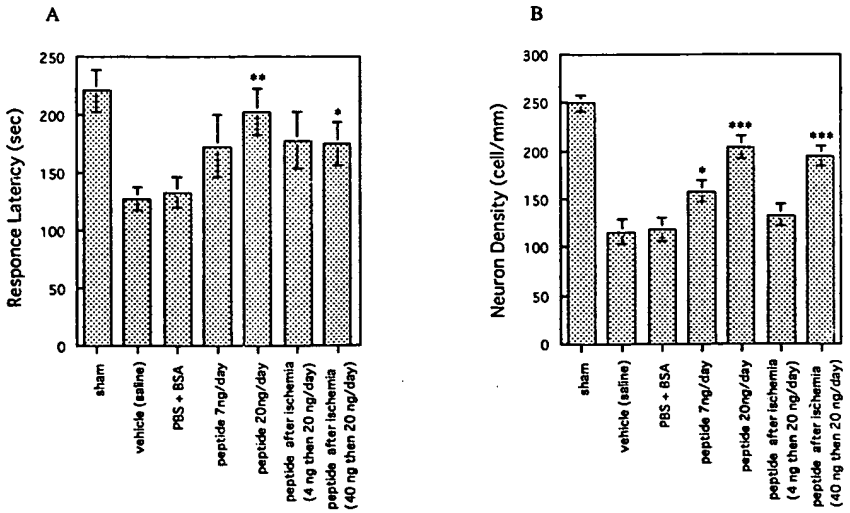

FIG. 4. Preventive effect of the 18-mer peptides (**A**) on learning disability in 3-min ischemic gerbils and (**B**) on the number of viable hippocampal CA1 neurons. Peptide treatment, starting either 2 h before or immediately after ischemia, prevented significantly the occurrence of ischemia-induced learning disability and hippocampal CA1 neuron loss in a dose-dependent manner. Data are mean ± SE (n = 7–11). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

128 ± 10 s ($p < 0.01$); and PBS/BSA infusion, 133 ± 13 s ($p < 0.05$) (Fig. 4A). Subsequent histological examinations revealed that the peptide treatment rescued many ischemic CA1 neurons that were destined to degenerate without the treatment: 20 ng/day of peptide treatment, 204 ± 12 cells/mm; vehicle infusion, 116 ± 13 cells/mm ($p < 0.001$); and PBS/BSA infusion, 119 ± 11 cells/mm ($p < 0.001$) (Fig. 4B). Our previous study showed that infusion of prosaposin at a dose of 240 ng/day exhibits a neuroprotective action similar to that of the 18-mer peptide (Sano et al., 1994), although the molar concentration of the 18-mer peptide used in this study is approximately three times higher than the prosaposin concentration. The 18-mer peptide, when administered immediately after ischemic insult, was also significantly effective in preventing ischemia-induced learning disability and neuronal death (Fig. 4). This finding suggested that the 18-mer peptide is capable of reversing or stopping the intracellular apoptotic processes triggered by transient forebrain ischemia (Nitatori et al., 1995).

Although the molecular mechanisms underlying the neurotrophic action of the 18-mer peptide are not clear at present, O'Brien et al. (1994) suggested the presence of a putative prosaposin receptor on the surface of neuroblastoma cells. They further demonstrated that prosaposin binding to the receptor stimulated tyrosine phosphorylation of cytoplasmic proteins, initiating a signal transduction cascade induced by prosaposin (O'Brien et al., 1995). The 18-mer peptide may also act on hippocampal CA1 neurons in vitro and in vivo through its binding to prosaposin receptor.

In conclusion, we have ascribed the neurotrophic action of an 18-mer peptide sequence in prosaposin and raise the possibility that the peptide or its analogue can be used as a tool for the treatment of ischemic cerebrovascular diseases.

Acknowledgment: We are grateful to Ms. Kazumi Matsumoto for her technical assistance. This work was supported by a Grant-in-Aid for Scientific Research on Priority Areas, from the Ministry of Education, Science, Sports and Culture of Japan (to A.S.).

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